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Endocannabinoid Metabolomics: A Novel Liquid Chromatography–Mass Spectrometry Reagent for Fatty Acid Analysis

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Abstract

We have synthesized 4,4-dimethoxyoxazoline derivatives of several fatty acids associated with the endocannabinoid metabolome using tris(hydroxymethyl)aminomethane in a 1-step reaction by microwave irradiation. The derivatization incorporates a nitrogen into the final product, which allows for improved detection by liquid chromatography–mass spectrometry in positive atmospheric pressure chemical ionization (APCI) mode. Palmitic and oleic acid derivatives show a 200-fold increase in sensitivity compared with the free acids when analyzed in negative-mode APCI. In addition to improving sensitivity, the oxazoline derivatization creates a similar ionization response for the fatty acids tested, which simplifies their quantitation. Fatty acid oxazoline derivatives can be detected using the same conditions optimized for the endocannabinoids, which allows for a simultaneous quantitation of the entire endocannabinoid metabolome.

Keywords: fatty acids, LC-MS, metabolome, oxazoline derivatives, microwave synthesis

Introduction

The endocannabinoid system is modulated by several families of lipid and lipidlike signaling molecules that collectively constitute the endocannabinoid metabolome. Endocannabinoids are known to be biosynthesized and biotransformed by several enzymes, leading to a variety of enzymatic products and intermediates, many of which appear to play a role in the modulation of endocannabinoid-related physiological functions. We have sought to introduce highly sensitive analytical techniques based on liquid chromatography–mass spectrometry (LC-MS) for the characterization of the endocannabinoid metabolome, including the quantitative determination of its components and the discovery of novel, as-yet-unidentified members of this family of lipid modulators. Many of the endocannabinoid ligands are biosynthesized within the cell membrane, which serves as a reservoir of fatty acids that are used as precursors for these biologically important signaling molecules. Therefore, the quantitative measurement of fatty acids remains an important component in the characterization of the endocannabinoid metabolome.

A popular method for endocannabinoid analysis is LC/MS using atmospheric pressure chemical ionization (APCI).[1,2] However, it is known that free fatty acids and methyl ester derivatives exhibit poor sensitivity in LC-APCI-MS analysis.[3] Attempts to develop a comprehensive quantitative method for the determination of endocannabinoids and their precursor fatty acids were hampered by the wide variability and lack of sensitivity for many of the fatty acids. We approached this problem by transforming the individual fatty acids into their respective 2-oxazoline derivatives. Such derivatization is commonly used in gas chromatography–mass spectrometry (GC-MS) analysis but has not yet been applied to LC-MS analysis.[4]

Oxazoline derivatives in LC-MS are useful because they incorporate a basic protonatable nitrogen atom, which is necessary for positive-mode APCI-MS. Conversely, free fatty acids will ionize in only negative-mode APCI, a method that is less sensitive and depends on the nature of the fatty acid being analyzed. Analysis of the oxazoline derivatives in positive mode allows for their simultaneous detection with other endocannabinoids, simplifying both the liquid chromatographic conditions and mass spectral analysis. To test this concept, we synthesized 4,4-bis(hydroxymethyl)-2-oxazoline derivatives of 3 fatty acids. The preparation of these compounds was accomplished in 1 step by microwave irradiation using an excess of the reagent tris(hydroxymethyl)aminomethane (tris). The simplified fragmentation pattern of the oxazoline derivatives by MS/MS creates strong selected reaction monitoring (SRM) transitions that can be exploited to increase the signal response by up to 200 times compared with underivatized fatty acids.

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Experimental Procedures

General

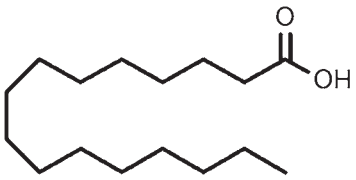
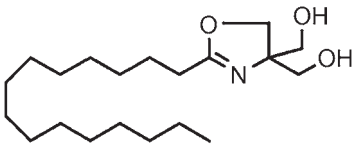
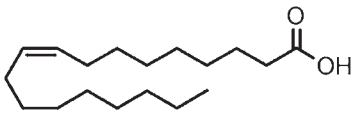
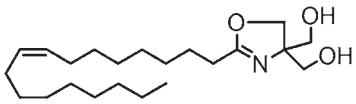
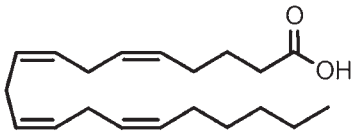
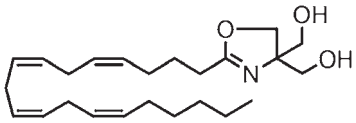
Arachidonic acid was obtained from NuChek Prep (Elysian, MN), while oleic acid, palmitic acid, tris, and other reagents were obtained from Sigma Aldrich (St Louis, MO) and had 99% or greater purity. All high-performance liquid chromatography (HPLC)-grade solvents were obtained from Fisher Scientific (Fairlawn, NJ). Reactions were performed using a CEM Discover microwave synthesizer (Matthews, NC) in a 10-mL glass vessel and monitored by thin-layer chromatography (TLC) (EMD Chemicals precoated glass TLC plates, Si60, F_{254}, 0.25-mm layer thickness) by eluting with acetone:hexane (3:7, vol/vol). Detection of products on TLC was performed by dipping in 5% phosphomolybdic acid (PMA) in 95% ethanol. Crude products were purified on silica gel using a Biotage SP1 Flash Chromatography (Uppsala, Sweden) system and acetone:hexane as the gradient mixture of eluents. The products were characterized by a Varian Inova (Palo Alto, CA) 500 (500 MHz) nuclear magnetic resonance (NMR) instrument at 25°C in $CDCl_3$; chemical shifts are reported in ppm with tetramethyl silane as the internal standard. Products were analyzed using an Agilent 1100 system HPLC (Palo Alto, CA) and a Thermo Quantum Ultra triple quadrupole mass spectrometer (San Jose, CA).

Synthesis of Oxazoline Derivatives

The microwave reactions were performed for 30 minutes, and the reaction temperature was held constant at 230°C, with a maximum variable power of 275 W. Approximately 200 μmol of each acid was reacted with tris in a 1:5 molar ratio. After the reaction was complete, 20 mL of water was added and the reaction mixture extracted with ethyl acetate (3 × 20 mL). The combined organic layers were dried over $MgSO_4$ and the solvent evaporated on RotaVapor (Buchi, Flawil, Switzerland) under vacuum. The crude product was analyzed by TLC (30% acetone in hexane) and NMR. The reaction mixtures were over 95% pure. These were purified by flash chromatography on silica gel using a 2-solvent system in which solvent A was hexane and solvent B was acetone; the gradient was allowed to run from 15% to 100% solvent B over 10 column volumes. The compounds eluted at ~60%B and were identified by NMR, as follows:

(4-Hydroxymethyl-2-pentadecyl-4,5-dihydro-oxazol-4-yl)-methanol ^{1}H NMR (500 MHz, $CDCl_3$) δ 4.18 (s, 2H), 3.69, 3.61 (ABq, J = 8.5 Hz, 4H), 2.31 (t, J = 8.0 Hz, 2H), 1.65 (quint, J = 7.0 Hz, 2H), 1.30 (m, 24H), 0.89 (t, J = 6.5 Hz, 3H) (Table 1, entry 1), [2-((Z)-heptadec-8-enyl)-4-hydroxymethyl-4,5-dihydro-oxazol-4-yl]-methanol ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.38 (m, 2H), 4.17 (s, 2H), 3.69, 3.61 (ABq, J = 11.5 Hz, 4H), 2.31 (t, J = 8.0 Hz, 2H), 2.02 (q, J = 6.5 Hz, 4H), 1.65 (quint, J = 7.5 Hz, 2H), 1.32 (m, 20H), 0.89 (t, J = 6.5 Hz, 3H) (Table 1, entry 2), [4-hydroxymethyl-2-((4Z,7Z,10Z,13Z)-nonadeca-4,7,10,13-tetraenyl)-4,5-dihydro-oxazol-4-yl]-methanol ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.41 (m, 7H), 4.19 (s, 2H), 3.69, 3.61 (ABq, J = 11.0, 4H), 2.85 (m, 6H), 2.34 (t, J = 7.5 Hz, 2H), 2.16 (m, 2H), 2.07 (m, 2H), 1.75 (quint, J = 7.5 Hz, 2H), 1.37 (m, 6H), 0.90 (t, J = 6.5 Hz, 3H) (Table 1, entry 3).

Table 1. Microwave Promoted Oxazoline Derivatives from Fatty Acids

Entry	Fatty Acid	Time (min)/ Temp (°C)	Product	Isolated Yield
1	 Palmitic Acid	30/230	 [1]	91%
2	 Oleic Acid	30/230	 [2]	95%
3	 Arachidonic Acid	30/230	 [3]	92%

LC-MS Conditions

Serial dilutions were made from purified stock solutions of the 3 derivatives and from the commercially obtained fatty acid standards. Their signal intensities at various concentrations were recorded by LC-MS on an Agilent 1100 HPLC and a Thermo Quantum Ultra triple quadrupole mass spectrometer. Two reverse-phase gradient methods were developed. The aqueous phase for the underivatized fatty acids was 10 mM ammonium acetate, pH 7.3, while for the oxazoline derivatives it was 0.1% formic acid. The organic phase for both methods was methanol. Initial gradient conditions were 10%B, and after 1 minute, the gradient ramped to 60%B and increased linearly to 75%B over 9 minutes. At 10 minutes, the gradient increased to 95%B and was held isocratically for 2.5 minutes. At 12.5 minutes, the gradient returned to 10%B, and the run ended at 15 minutes. All compounds were separated with a flow rate of 500 µL/min on an Agilent Zorbax SB-CN column, 2.1 × 50 mm, 5-µ particle size. The 3 underivatized acids were ionized in APCI negative mode, and the derivatives were ionized in APCI positive mode. The reagent gas used was N_2, while the vaporizer and capillary temperatures were 400°C and 250°C, respectively; the coronal discharge current was set at 6 µa. The collision pressure was 1 mTorr, the sheath gas and auxiliary gas were set at 25 and 5 respectively, and the source collision induced dissociation (CID) was set at 8. SRM transitions used in the tandem MS analysis are listed in Table 2.

Results and Discussion

Chemical derivatization of fatty acids for improved sensitivity of detection has been practiced for several decades.[5,6] Traditionally, carboxylic acids are converted to their methyl esters for improved volatility in GC-MS. Another group of fatty acid derivatives are the 2-oxazolines, which, under MS conditions, have been shown to fragment into several specific species. This mode of fragmentation greatly simplifies the electron impact mass spectrum.[7] Specifically, 2-alkenyl-4,4-dimethyloxazoline is used to determine double bond location in unsaturated fatty acids by GC-MS.[8] The oxazoline derivatives of carboxylic acids encompass a heterocyclic ring nitrogen, which could provide a site for protonation when ionized by APCI+. The simplified fragmentation pattern of the oxazoline derivatives also provides better SRM transitions that can be monitored by tandem mass spectroscopy, leading to improved sensitivity of detection. This enhanced sensitivity was confirmed by the MS analysis of the oxazoline fatty acid analogs reported here.

Table 2. SRM Transition and Collision Energy for Acids and Derivatives*

Compound	SRM Transition	Collision Energy
Palmitic acid	255.18→237.25	25
Oleic acid	281.15→263.15	25
Arachidonic acid	303.13→259.17	15
Palmitic-oxazoline	342.29→294.06	29
Oleic-oxazoline	368.30→158.05	42
Arachidonic-oxazoline	390.27→158.05	42

*SRM indicates selected reaction monitoring.

Synthesis of Oxazoline Fatty Acid Analogs

Synthesis of 2-oxazoline derivatives can be accomplished by a variety of methods.[9-11] Typically, β-amino alcohols, such as 2-amino-2-methylpropanol, are first allowed to react with the carboxylic acid to form an amide, followed by ring cyclization to produce the substituted 2-oxazolines. It has been shown that the synthesis of 2-oxazolines can be performed solvent-free via microwave irradiation, a technique that is attractive because of its speed, ease, and green chemistry implications.[12]

Several reaction conditions were explored, including the use of high-boiling-point aprotic solvents such as dimethyl sulfoxide (DMSO) and 1-methyl-2-pyrrolidone. However, optimal results were obtained under solvent-free conditions. The first derivative, 4,4-dimethyl-2-alkenyl-oxazoline from 2-amino-2-methylpropanol, was obtained in less than the 90% minimum we had designated as a target yield for this analytical method. Although the starting material had undergone a complete reaction in the first step, there remained a large percentage of uncyclized amide intermediate. Conversion of the amide to the cyclized oxazoline product was significantly improved with the use of other amino alcohols such as 1-amino-2-methylpropanol and ethanolamine for the derivatization. The best results were obtained when tris was used to provide the 4,4-bis(hydroxymethyl)-2-alkenyl-oxazoline derivative of the corresponding carboxylic acid. Following the reaction in Figure 1, we successfully synthesized the oxazoline derivatives of palmitic, oleic, and arachidonic acids (Table 1, entries 1, 2, and 3). The crude reaction mixture showed complete conversion to a single product in 95% purity based on TLC/NMR analysis. After

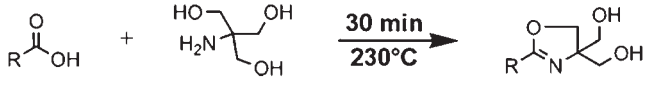


Figure 1. General reaction scheme for the conversion of a carboxylic acid to a bis(hydroxymethyl) oxazoline derivative using tris(hydroxymethyl)aminomethane. Three derivatives were synthesized using a microwave-assisted solvent-free method performed at 230°C for 30 minutes.

purification using flash chromatography, the isolated yield for each derivative was greater than 90%, as shown in Table 1.

LC/MS Analysis of Oxazoline Fatty Acid Derivatives

The typical concentration range for an endocannabinoid calibration curve is 5 pg/µL to 500 pg/µL. However, palmitic acid and oleic acid are not detectable in this range. As can be seen in Figure 2A, at the 500 pg/µL level only arachidonic acid can be detected, while Figure 2B shows that the response of the palmitic and oleic oxazoline derivatives is significantly more sensitive compared to the free acids, considering that the free acids were undetectable at this concentration. At 500 pg/µL, the MS spectra of the palmitic and oleic oxazoline derivatives exhibit signal-to-noise ratios of 13 414 and 2167, respectively. The signal-to-noise ratio for arachidonic acid is also improved, although to a lesser extent, by a factor of 5, from 1638 to 9036, when it is analyzed as the oxazoline derivative.

The observed enhancement in signal intensity for the oxazoline derivatives drastically expands the limits of quantitation for palmitic and oleic acids and significantly enhances that of arachidonic acid. Figures 3A and 3B show a comparison of the concentration levels of derivatized and underivatized acids that are necessary to reach approximately the same limit of quantitation. The 1000 pg/µL level necessary for detecting palmitic and oleic acids is up to 200 times greater than their derivatized forms at 5 pg/µL, while the improvement for derivatized arachidonic acid is twice that of the underivatized form. The improved sensitivity of their derivatives allows us to monitor all these fatty acids at levels comparable to those used in the analysis of other

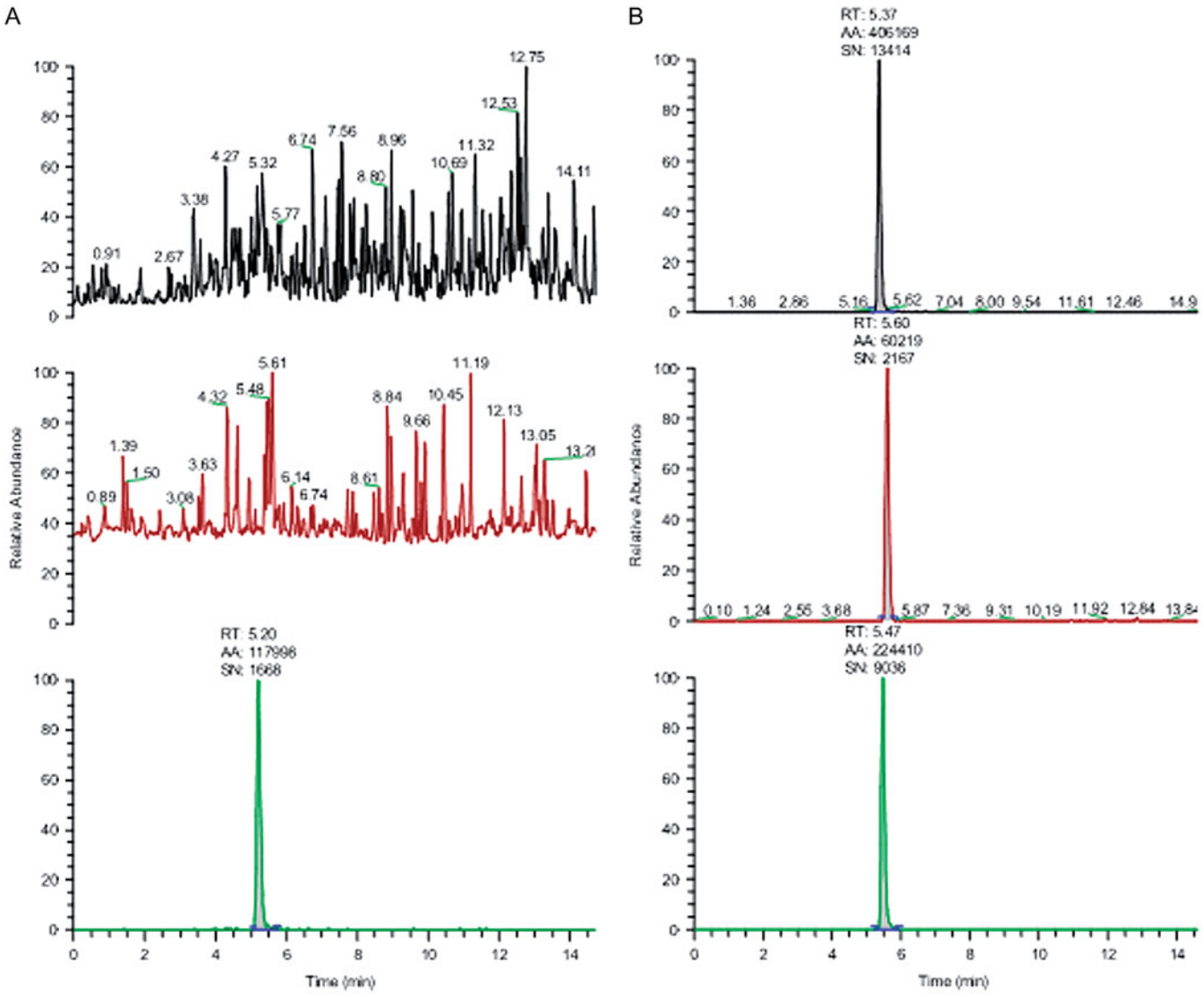


Figure 2. (A) Normalized extracted ion chromatograms of palmitic (top), oleic (middle), and arachidonic acid (bottom) at 500 pg/µL. Mobile phase consisted of 10mM ammonium acetate:methanol. (B) Normalized extracted ion chromatograms of palmitoyl-tris (top), oleoyl-tris (middle), and arachidonoyl-tris (bottom) derivatives at 500 pg/µL. Mobile phase consisted of 0.1% formic acid/methanol. Tris indicates tris(hydroxymethyl)aminomethane.

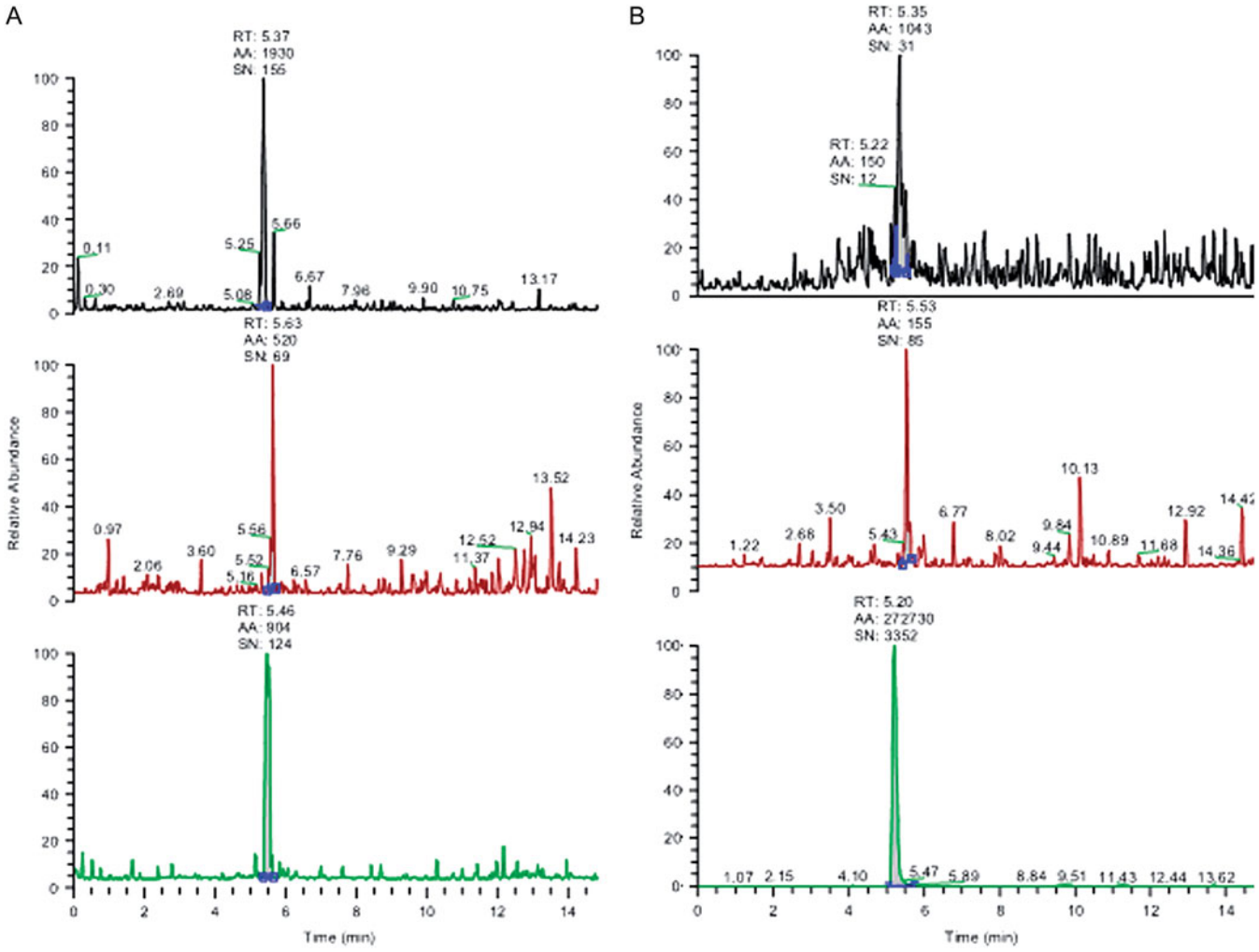


Figure 3. (A) Normalized extracted ion chromatograms of palmitoyl-tris (top), oleoyl-tris (middle), and arachidonoyl-tris (bottom) derivatives at 5 pg/uL. Mobile phase consisted of 0.1% formic acid/methanol. (B) Normalized extracted ion chromatograms of palmitic (top), oleic (middle), and arachidonic acid (bottom) at 1000 pg/uL. Mobile phase consisted of 10mM ammonium acetate: methanol. Tris indicates tris(hydroxymethyl)aminomethane.

endocannabinoids, such as anandamide, in biological samples. Previously, the underivatized free fatty acids could be analyzed in only a separate run in APCI mode because different solvent systems were required for their optimal ionization. However, the incorporation of a nitrogen atom into the derivatized products allows these compounds to be ionized and analyzed in positive-mode APCI, like the other endocannabinoids. Overall, this approach to fatty acid determination simplifies and speeds up the analysis of the endocannabinoid metabolome, because now all compounds within this group can be quantified using the same method.

Conclusions

As part of our effort to develop sensitive methods for the quantitative determination of the endocannabinoid metabolome, this preliminary study has achieved 2 goals. First, for the derivatization of fatty acids we developed microwave reaction conditions that yield near-complete conversion to the desired 2-oxazoline product in 1 step without the use of solvents. Second, the MS analysis demonstrated that the 2-oxazoline derivatives produced a response that was much better than that of the underivatized acids by LC-APCI-MS. Approximately 200-fold improvements in limit of quantitation (LOQ) for palmitic and oleic acids were achieved, while arachidonic acid was enhanced by 2-fold. The derivatization procedure eliminates the need to analyze the free acids using an ionization mode different from that used for the rest of the endocannabinoids in the metabolome sample. Additionally, the derivatization procedure had a leveling effect on the 3 fatty acids' ionization efficiency. Whereas the underivatized acids show a wide response in signal intensity at the same concentration, the oxazoline derivatives all have a similar response and at much lower concentrations.

The next step in this project will be to determine whether the microwave reaction can be specific enough to derivatize

fatty acids in the presence of other endocannabinoid ligands, most notably anandamide and 2-arachidonoyl glycerol. This will allow us to quantify all components of the endocannabinoid metabolome in a single experiment and gain a better understanding of the endocannabinoid system's response to physiological change.

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